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DEGRADATION OF OIL BY YEAST AND FILAMENTOUS FUNGI IN ARCTIC ENV--ETC(U)

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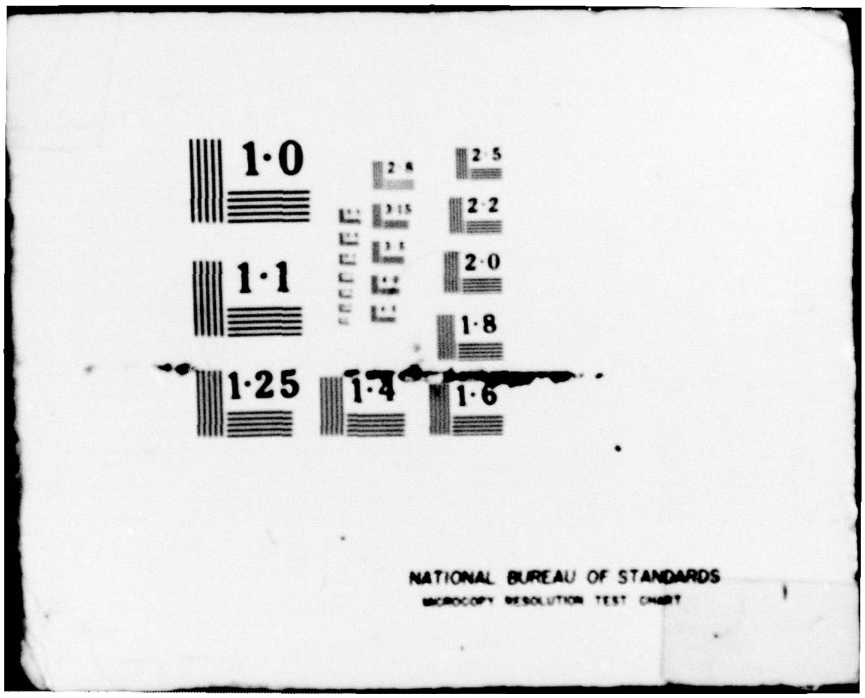
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Approximately 200 yeast isolates from Alaskan tundra, Arctic regions of the North Atlantic and other low temperature environments were screened for low temperature metabolism of hydrocarbons. None of the cultures examined were comparable to Candida lipolytica or Candida maltosa at elevated or low temperatures. Studies of these two yeast have demonstrated that both are capable of oxidizing a range of aromatic hydrocarbons including naphthalene and benzopyrene. Candida maltosa generally grows better on hydrocarbons than C. lipolytica. However, C. lipolytica appears to immobilize large quantities

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20. of hydrocarbon. Differences in the uptake of non-growth supporting hydrocarbons have been observed between these two yeasts.

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Contract No. N00014-76-C-1058

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FINAL REPORT

DEGRADATION OF OIL BY YEAST AND FILAMENTOUS FUNGI IN ARCTIC ENVIRONMENTS

by

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30 November 1979

This research was supported in part by the Office of Naval Research, Microbiology Program, Naval Biology Project, under contract, No. N00014-76-C-1058.

Scope of the Work

The principal objective of this study was to evaluate low temperature degradation of oil by yeasts and to examine their potential for facilitating the biodegradation of oil at low temperatures. During the contract period, 1 September 1976-1 September 1979, approximately 200 yeast isolates from Alaskan tundra samples, Arctic regions of the North Sea and other low temperatures were physiologically characterized and examined for low temperature hydrocarbon metabolism. Cultures of *Candida maltosa* and *Candida lipolytica* were studied extensively to establish the general physiological aspects of low temperature hydrocarbon metabolism in yeast and fungi.

Research Accomplished

During the initial phase of the contract 40 yeast isolates from cold regions of the North Sea (5 stations between 65°13'N, 07°52'E; 68°56'N, 13°32'E) were examined. The isolates were classified into morphological and physiological groupings and identified according to Lodder (1970), Barnett and Parkhurst (1974). *Debaryomyces hansenii*, *Candida guilliermondii* were the most common yeast isolates. Both these yeasts have been reported to utilize hydrocarbons (Scheda and Bos 1966). Isolates of *Rhodotorula* and several additional species of *Candida* were obtained. Representatives of the most common groups were examined for growth on hexadecane at 22-24°C and subsequently for growth on glucose and hexadecane at 8°C. Several isolates grew slowly on hexadecane at room temperature. Most cultures were capable of only slow growth on glucose at low temperatures, with doubling times approaching 48-60 hrs

following an initial lag of 3-5 days. With hexadecane at low temperatures, no growth was obtained with these isolates. The nature of the semisolid hydrocarbon at this temperature may prohibit transport. The very active hydrocarbonoclastic yeasts, *Candida lipolytica* and *C. maltosa*, are active at this temperature with the hexadecane substrate. The psychrotolerant *C. lipolytica* grows much better than the mesophilic *C. maltosa*. Hydrocarbon mixtures containing pristane (tetramethylpentadecane) were developed and have proved to be extremely useful in the study of low temperature hydrocarbon utilization since they do not undergo solidification at temperatures above 0°C. The most useful mixture contains 10 ml hexadecane, 10 ml tetradecane, 10 ml pristane and 1 g naphthalene.

Samples of Alaskan high center and low center tundra soils from Summers 1975 and 1976 were obtained from Dr. Ronald M. Atlas, University of Louisville. The samples were subjected to standard enumeration techniques using mycological agar (Difco) with chloramphenicol. Cultures were incubated at 5°C and 20°C to determine the dominant yeast at each temperature. Enrichment cultures contained hexadecane or Louisiana crude oil in a yeast nitrogen base broth were prepared from diluted tundra material. One series of enrichment flasks was prepared with chloramphenicol to inhibit bacteria, the other received no chloramphenicol. Cultures without chloramphenicol showed signs of microbial degradation after 14 days at 20°C. Numerous filamentous fungi and yeasts were isolated from these samples. Extensive mycelial mats were formed in the hexadecane cultures from all four sites. Only the high center 1976 produced extensive mycelial development on the crude oil. Bacterial cultures were isolated from all systems suggesting a possible metabolism of the hydrocarbons by the entire microbial population.

Systems with chloramphenicol grew more slowly and only slightly emulsified the oil or hexadecane at 14 days, further suggesting the combined role of bacteria, yeast, and filamentous fungi in low temperature oil degradation.

The predominant yeast isolates were members of the genera *Cryptococcus*, *Rhodotorula*, and *Leucosporidium* and were not capable of growth on single hydrocarbons at either room temperature or 5-10°C. They would appear to be surviving and perhaps metabolizing the by-products of bacterial hydrocarbon utilization in mixed culture systems.

A number of yeast isolates were obtained from North Sea waters during February 1978 (supplied by Dr. W. Gunkel, Biologische Anstalt, Helgoland, Germany). Physiological comparisons of these isolates with previous isolates from the North Sea again demonstrated limited mesophilic hydrocarbon utilization but no psychrotolerant hydrocarbon metabolism.

Samples of oil (surface films and mousse) were collected by Dr. D. G. Ahearn on the rocky shore of Portsall, Normandy, France approximately one mile from the wreck of the Amoco Cadiz. These samples have been introduced into marine broth (Difco) and mycological broth as well as a seasalts solution and incubated 15°C to 20°C. Periodically, developing microorganisms were isolated and screened by a gas chromatographic procedure to detect utilization of hydrocarbons in a hydrocarbon mixture. The isolation of the yeasts from the oil samples suggested successional development of species. *Rhodotorula rubra* was isolated sporadically in the highest numbers during the first 2-3 months of study, particularly from the surface film samples. This species has been noted

in bloom occurrences in various marine waters, but representative Amoco Cadiz and North Sea isolates gave negligible growth on hexadecane and crude oil fractions. During the first three months only an occasional isolate of C. tropicalis, C. guilliermondii and Debaryomyces hansenii was obtained from the brown-black oil and the mousse samples. After three months R. rubra was no longer isolated from the samples and isolates of C. tropicalis and C. guilliermondii were obtained commonly. These latter species in contrast to R. rubra readily utilized hexadecane as a sole source of carbon for growth at 22-24°C. None of the yeasts, including the single isolate of C. lipolytica, however, gave good oil emulsification or utilization comparable to that found for isolates of C. lipolytica and C. maltosa from other sources (Ahearn et al. 1976). Two morphologically distinguished cultures of Pseudomonas which grew well in the broth media with good oil emulsification were isolated sporadically from all except the surface film samples.

All yeasts isolated from Amoco Cadiz oil represented species previously isolated from waters of the North Sea. The yeasts, apparently in low numbers, showed a successional development in the oil to species with greater hydrocarbonoclastic activity. Candida guilliermondii, a yeast with moderate hydrocarbonoclastic activities, was the second most common isolate obtained in 1976, whereas only several atypical isolates initially identified as Candida sp. were obtained in 1964-66. In 1976, the incidence of Aureobasidium pullulans appeared reduced. Meyers et al. (1968) found yeasts at all of twelve stations in 99% of 84 samples. The majority of samples contained between 35-50 cells/L with the maximum density of >3,000 cells/L. In 1976, yeasts were isolated from 100% of the surface samples collected at the 35 stations and from 28 of the 35 samples collected at 10 m. Yeast densities at the surface averaged 76 cells/L and 35 cells/L at 10 m.

Relatively few species of fungi were isolated from the Amoco Cadiz oil. The direct sampling onto agar plates of all samples gave only a few colonies indicating that fungal populations were <5 colony forming units per 100 ml representing no more than two species. When these oil samples were vigorously agitated in a Tween 80 solution, densities in some samples ranged to 57 cells/ml and others yielded up to five different species. The greatest variety of species was obtained from the surface films.

In comparison to surface films and water samples examined in earlier work, the crude oil appeared selective and possibly inhibitory to normal marine yeast flora. Certain volatile hydrocarbons, dependent upon concentration, may be cidal for yeasts (Ahearn et al. 1971). The Iranian oil, which contained about 30% naphthalenes, has proven inhibitory to representative isolates of Debaryomyces hansenii, the most common yeast in North Sea waters in simple spot tests on nutrient agar. The presence of odorous volatile oil fractions at the shore adjacent to the wreck was quite noticeable even 10 days after the spill. The high concentrations of these volatile fractions may have markedly reduced the densities and species of yeasts brought into contact with the oil. The effects of various crude oils on the role of marine occurring yeasts in the recycling of nutrients are poorly understood.

Examination of a number of yeast and filamentous fungi from low temperature environments have not readily demonstrated rapid degradation by the mycoflora of most natural environments. However, processes active in chronically oil-polluted low temperature environments may select for organism considerably different from those recovered in this study.

A second facet of the work involved the detailed examination of hydrocarbon metabolism by the psychrotolerant yeast C. lipolytica and comparison to C. maltosa, a distinctly mesophilic hydrocarbon utilizer.

Base line studies of mixed hydrocarbon utilization at 20°C have consistently shown that both C. maltosa and C. lipolytica are capable of reducing concentrations of naphthalene and biphenyl in hydrocarbon mixtures composed of methylcyclohexane ethylbenzene, naphthalene, biphenyl, tetradecane, hexadecane, and eicosane. A second hydrocarbon mixture was developed for low temperature studies. This oil contains equal parts of tetradecane, hexadecane and pristane and .03 gm of naphthalene/ml of mixture. Cultures of R-42 and 37-1 have been shown to metabolize the n-alkane, aromatic, and branched chain components of this mixture. Preliminary studies at 5°C showed that selected isolates of C. lipolytica grow well on this mixture and that over 50% of the mixture was no longer recoverable. All fractions of the oil were utilized at this temperature. Naphthalene and pristane were recovered from yeast cultures at levels of only 25 and 55% of the uninoculated control flasks.

Yeast growth and utilization of hydrocarbons at 5°C is dependent upon the physiological state of the inoculum (e.g. previous growth substrate) and inoculum size. With prior growth on glycerol the lag phase is reduced and the initial rate of hydrocarbon uptake is increased as contrasted to glucose or tetradecane grown cells.

A simple technique to quantitatively separate yeast cells from spent hydrocarbon medium was developed. This procedure permits analysis of uptake of hydrocarbon substrates which fail to support growth. The effects of these compounds, principally naphthalene, on the utilization of hydrocarbon growth substrates were demonstrated.

Comparison of growth kinetics of the hydrocarbon utilizing yeast Candida lipolytica and C. maltosa with glycerol as a carbon source have indicated that both yeast have similar growth rates. However, on glucose C. maltosa grew much more rapidly (doubling time 1.4 vs. 4.1). Both organisms grew slower on hydrocarbon substrates but growth was always more rapid for C. maltosa. Growth and yield for C. lipolytica were best on hexadecane, followed by pristane and tetradecane. Candida maltosa grew best on tetradecane but gave similar yield but slower growth with hexadecane. Incorporation of naphthalene into tetradecane affected both the yield and growth rates of both organisms. A comparison of naphthalene concentration and yield on tetradecane illustrated differences between the two yeasts. A general reduction in yield was found with increasing naphthalene concentration for C. maltosa. In C. lipolytica yield increased with increases in naphthalene concentration up to 100 ppm, remained constant to 200 ppm, then declined at concentrations above 200 ppm. Comparison of cell crop and hydrocarbon losses from a mixed hydrocarbon substrate demonstrated greater hydrocarbon losses with C. lipolytica even though greater yield was obtained with C. maltosa.

Detailed studies of uptake and binding of single hydrocarbons and mixtures have noted several additional differences between these two yeasts (Crow et al. in press). Pristane uptake was concentration dependent in C. maltosa but not in C. lipolytica. The presence of glucose suppresses hydrocarbon utilization in C. maltosa particularly depressing the loss of non growth supporting compounds naphthalene and pristane. Uptake study verified a similar depression of hydrocarbon uptake by C. maltosa at low temperature.

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Major Accomplishments:

1) Observation of naphthalene and pristane utilization from mixed hydrocarbon substrates by yeasts.

2) Verification of pristane as a growth substrate for C. lipolytica.

3) Established a useful mechanism for studying the growth kinetics of hydrocarbon grown yeasts.

4) Established several major differences in the responses of the yeast to low temperature and hydrocarbon uptake.

5) In collaboration with Dr. Carl Cerniglia, University of Texas at Austin, we have demonstrated the capacity of both yeasts to oxidize a wide range of aromatic hydrocarbons.

6) Demonstrated the capacity of Candida lipolytica to degrade hydrocarbons at low temperature.

Presentations and Publications on the Contract

The uptake of Aromatic and Branched Chain Hydrocarbons by Yeast. 1980. S. A. Crow, S. L. Bell, D. G. Ahearn. Botanica Marina Vo. XXII, in press.

Yeasts from the North Sea and Amoco Cadiz Oil. 1980. D. G. Ahearn and S. A. Crow. Botanica Marina Vol. XXII, in press.

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